Characterization of Alkylmethoxypyrazines Contributing to Earthy/Bell Pepper Flavor in Farmstead Cheddar Cheese

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ABSTRACT: Farmstead Cheddar cheeses with natural bandage wrappings have a distinctive flavor profile that is appealing to many consumers. An earthy/bell pepper (EBP) flavor has been previously recognized in some of these cheeses. This study characterized the alkylmethoxypyrazine compounds causing EBP flavor in Farmstead Cheddar cheeses. Eight cheeses were divided into inner, outer, rind, and wrapper sections, and tested for descriptive sensory and instrumental analyses. To assess reproducibility of EBP flavor, cheeses from the same facilities were purchased and tested after 6 and 12 mo. EBP flavor was detected in four out of 8 Farmstead Cheddar cheeses by a trained sensory panel. 2-sec-butyl-3-methoxypyrazine and 2-isopropyl-3-methoxypyrazine were identified as the main sources of EBP flavor in these cheeses by GC/O and GC/MS. In general, those alkylmethoxypyrazines were prevalent in the wrapper (106 to 730 ppb) and rind (39 to 444 ppb) sections of the cheeses. They were either not detected in inner and outer sections of the cheeses or were present at low concentrations. These results suggest that 2-sec-butyl-3-methoxypyrazine and 2-isopropyl-3-methoxypyrazine are formed near the surface of the cheeses and migrate into the cheese during ripening. Threshold values in water and whole milk were 1 and 16 ppt for 2-sec-butyl-3-methoxypyrazine, and 0.4 and 2.3 ppt for 2-isopropyl-3-methoxypyrazine, respectively. Sensory analysis of mild Cheddar cheese model systems confirmed that direct addition of those individual alkylmethoxypyrazines (0.4 to 20 ppb) resulted in EBP flavor.

Keywords: alkylmethoxypyrazines, cheese flavor, earthy/bell pepper flavor

Introduction

Farmstead Cheddar cheeses with natural bandage wrappings have a distinct flavor profile that is appealing to many consumers (Lieberman 2007). A Farmstead cheese is defined as any type of cheese that is manufactured at the same location as the cows are milked (Lieberman 2007). Farmstead Cheddar cheeses are typically manufactured from unpasteurized milk and aged with a natural cloth or cheesecloth bandage for more than 1 y prior to marketing (Quickes Traditional, Devon, U.K.; personal communication). Studies have demonstrated that certain Farmstead Cheddar cheeses exhibit a characteristic earthy/bell pepper (EBP) flavor (Suriyaphan and others 2001). Drake and others (2001) developed a descriptive lexicon to characterize Cheddar cheese flavors, and reported that EBP flavor was one of several flavors that were not frequently observed in U.S. Cheddar cheeses. Among more than 250 Cheddar cheeses screened in their study, only one exhibited the EBP flavor: a British Farmstead Cheddar that was manufactured from raw milk and aged for 24 mo. Suriyaphan and others (2001) used gas chromatography coupled with olfactometry (GC/O) and mass spectrometry (GC/MS) to characterize the aroma properties of solvent extracts from a British Farmstead Cheddar cheese. They reported that the EBP flavor commonly found in this cheese was associated with alkylmethoxypyrazines such as 2-isopropyl-3-methoxypyrazine and 2-isobutyl-3-methoxypyrazine. They also hypothesized that these alkylmethoxypyrazines were formed on the surface of the cheeses and subsequently migrated into the cheese. However, instrumental detection limits and their sampling technique were not able to confirm this hypothesis.

Alkylpyrazines are found naturally in a variety of vegetables including bell peppers (Buttery and others 1969; Luning and others 1994), chili peppers (Pino and others 2006), potatoes (Buttery and others 1973), and green peas (Murray and others 1970). The herbaceous/bell pepper character of certain wines produced from Cabernet Sauvignon, Merlot, and Sauvignon Blanc grape varieties have been attributed to 2-isobutyl-3-methoxypyrazine, 2-sec-butyl-3-methoxypyrazine, and 2-isopropyl-3-methoxypyrazine (Allen and others 1994, 1995; Sala and others 2002). Chitwood and others (1983) reported that the "green" aroma of 3 bell pepper cultivars (*Capsicum)* was associated with 2-isobutyl-3-methoxypyrazine and 2-sec-butyl-3-methoxypyrazine.

Alkylpyrazines can be formed as byproducts of metabolic processes in some plants and microorganisms (Cheng and others 1991). For instance, alkylpyrazine mixtures similar to those produced in Maillard reactions have been identified in fermented cocoa (Gill and others 1984; Barel and others 1985), fermented soy (Liardon and Ledermann 1980), and cheese (Liardon and others 1982). 2-isopropyl-3-methoxypyrazine, which has been considered the source of musty-earthy off-flavor in eggs, milk, and fish, has been identified as a metabolite of *Pseudomonas perolans* and *Pseudomonas taetrolens* (Morgan 1976; McIver and Reineccius 1986; Gallois and others 1988; Cheng and Reineccius 1991; Cheng and

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others 1991). Brie and Camembert are cheeses that undergo surface ripening by *Pencillium* sp. and exhibit a characteristic musty-earthy flavor (Kosikowski 1982). Karahadian and others (1985a) identified several volatile compounds with earthy-musty notes that were produced by pure cultures of *Penicillium caseicolum* and *Penicillium camembert,* including a group of 8-carbon alcohols and corresponding ketones, 2-methylisoborneol, and 2-isopropyl-3-methoxypyrazine. All of these compounds were considered as potential contributors to the earthy-musty off-flavor of Camembert and Brie cheeses.

The use of instrumental analysis in conjunction with sensory techniques is an effective way to characterize flavor of foods. In general, links between sensory and instrumental analysis may be accomplished by 3 steps: (1) selection of flavor(s) using descriptive sensory analysis; (2) analysis of isolated volatile compounds using gas chromatography-olfactometry (GC/O) and gas chromatography-mass spectrometry (GC/MS); and (3) confirmation of aroma-active compounds via instrumental quantitation, threshold testing and sensory analysis of model systems (Drake and others 2006). The objectives of this study were to use this approach to characterize the volatile compounds contributing to EBP flavor in Farmstead Cheddar cheeses and to determine the concentrations and flavor impact of those compounds in cross sections of the cheeses.

Materials and Methods

Cheeses

Eight Farmstead Cheddar cheeses were used in this study (Table 1) and were purchased locally (Whole Foods; Raleigh, N.C., U.S.A.) or ordered online and shipped by overnight delivery on ice packs (www.idealcheese.com). A 7-kg cross-sectional wheel of Farmstead Cheddar cheese nr 1 was freshly cut from a 28-kg drum. The wheel was immediately divided into 4 wedges so that each had the same proportion of inner, outer, rind, and wrapper sections (Figure 1). The rind was defined as the dark-yellow side section of about 0.5 cm thick from the outer border of the cheese wedge; the inner was defined as the intersection of the cheese wedge 1 cm away from the top and bottom rind; the outer was defined as the section located 1 cm away from the side, top, and bottom rind (Figure 1). All 4 sections within a wedge, including the bandage wrapper, were evaluated by instrumental analysis. Inner and outer sections of the cheeses were evaluated using descriptive sensory analysis. To confirm the reproducibility of EBP flavor, additional wedges (2 kg) of Cheddar cheese nr 1 were subsequently purchased and analyzed after 6 and 12 mo (Whole Foods).

Farmstead Cheddar cheese nr 2 was purchased as a small 0.5-kg truckle. Due to the small size of this cheese, samples (inner, outer, rind, and wrapper) were obtained from the entire truckle rather than from different wedges. An additional 2-kg wedge of this cheese, which was freshly cut from a 28-kg drum, was purchased and analyzed after 1 y to access reproducibility of EBP flavor. Six other Farmstead Cheddar cheeses (Table 1; cheeses nr 3 to 8) were obtained as 2-kg wedges from 28-kg drums. An additional sample of Farmstead Cheddar cheese nr 8 was purchased as a 2-kg truckle. Inner, outer, and rind sections were sampled for sensory and instrumental analyses as previously described.

Table 1—Farmstead Cheddar cheeses selected for descriptive and instrumental analyses. Cheese wedges and cross sections were obtained from large 28-kg drums.

Cheese nr	Country of origin	January 2007	July 2007	January 2008
1	England	7-kg cross section	2-kg wedge	2-kg wedge
2	England	0.5-kg truckle	—	2-kg wedge
3	U.S.A.	—	2-kg wedge	2-kg wedge
4	Scotland	—	2-kg wedge	2-kg wedge
5	Australia	—	2-kg wedge	—
6	U.S.A.	—	2-kg wedge	—
7	England	—	—	2-kg wedge
8	England	—	—	2-kg truckle and 2-kg wedge

Descriptive sensory analysis of cheeses

A trained sensory panel ($n = 8$) with over 500 h experience in the descriptive sensory analysis of cheese evaluated the samples. Panelists were students and staff (ages 26 to 48 y) from the Dept. of Food, Bioprocessing and Nutrition Sciences at North Carolina State Univ., who were initially selected based on availability and ability to distinguish the basic tastes. Solutions of 250 ppt 2-sec-butyl-3-methoxypyrazine and 100 ppt 2-isopropyl-3-methoxypyrazine in water were provided to panelists during training along with cheeses with and without EBP to familiarize panelists with this flavor. Analysis of data collected from preliminary sessions confirmed that panelists could consistently identify and scale Cheddar cheese flavors including EBP.

Inner and outer sections of cheeses were evaluated for flavor and aroma using a previously established cheese sensory lexicon (Drake and others 2001). Cheeses were dispensed into lidded 58 mL soufflé cups with randomized 3-digit codes. Cheeses were tempered to 15 °C for sensory analysis. The panelists evaluated each sample in duplicate using a 15-point intensity scale, according to the Spectrum™ method (Meilgaard and others 1999). The panelists were provided with a mild Cheddar cheese along with a previously identified profile as a warm-up sample. Water and crackers were provided as palate cleansers between samples. Responses were collected with paper ballots or Compusense Five v4.6 (Compusense, Guelph, Ontario, Canada).

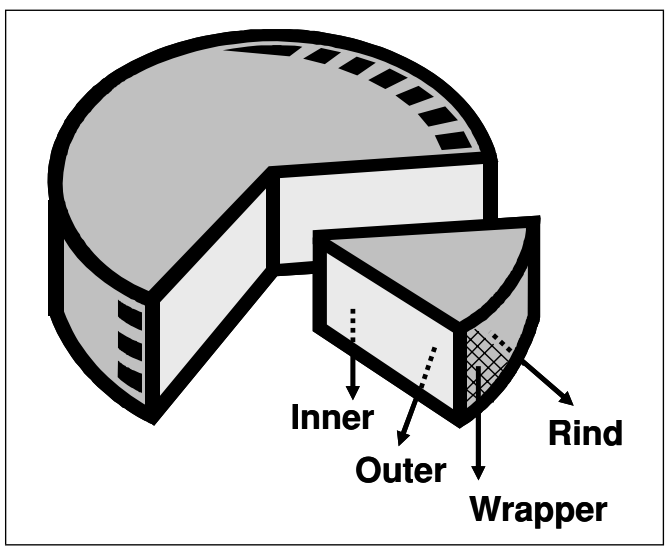

Figure 1—Schematic of Farmstead Cheddar cheese sampling used for descriptive sensory and instrumental analyses. Rind was the dark-yellow side section of about 0.5 cm thick from the outer border of the cheese wedge; inner was the intersection of the cheese wedge 1 cm away from the top and bottom rind; outer was the section located 1 cm away from the side, top, and bottom rind; wrapper was the bandage cloth used during cheese ripening.

Chemicals

Analyte standards of 2-sec-butyl-3-methoxypyrazine, 2-isopropyl-3-methoxypyrazine, and 2-ethylpyrazine (internal standard) were purchased from Sigma-Aldrich (St. Louis, Mo., U.S.A.). Methanol and ethanol served as solvents and were also obtained from Sigma-Aldrich. Stock solutions were prepared from the pure compounds diluted in absolute ethanol for sensory analysis or absolute methanol for instrumental analysis. Stock solutions were stored at −20 °C until use for a maximum of 1 mo.

Gas chromatography/mass spectrometry (GC/MS)

Cheese volatiles were isolated by solid phase microextraction (SPME) using a CTC Analytics combiPAL auto sampler (Zwingen, Switzerland). Five grams of grated cheese (or 16 pieces of 2.5 cm^2 wrapper) were placed into 20 mL clear screw cap vials (Microliter Analytical Supplies Inc., Suwanee, Ga., U.S.A.), and 9.7 ppb of 2-ethylpyrazine was added as the internal standard. A 3-phase 1 cm Stableflex 50/30 μm divinylbenzene/carbonex/PDMS (DVB/CAR/PDMS) fiber (Supelco, Bellefonte, Pa., U.S.A.) was exposed into the headspace above the sample at 40 °C with agitation at 250 rpm for 25 min. The fiber was inserted through the vial septa at 22 mm depth and remained in the headspace for 30 min before injection.

Extracted volatile components were analyzed on a 6890N GC/HP5973 mass selective detector (Agilent Technologies, Palo Alto, Calif., U.S.A.). The GC was equipped with a nonpolar capillary column (DB-5MS, 30 m length × 0.25 mm i.d. × 0.25 μm d_f; (J&W Scientific, Folsom, Calif., U.S.A.). Helium gas was used as carrier gas at a constant flow rate of 1 mL/min. Oven temperature was programmed to increase from 40 to 250 °C at a rate of 8 °C/min with initial and final hold times of 5 and 30 min, respectively. Mass selective detector conditions were as follows: capillary direct interface temperature, 250 °C; ionization energy, 70 eV; mass range, 35 to 350 m/z; EM Voltage (Atune +200 V); scan rate, 2.94 scans/s. All samples were run in triplicate using the splitless mode.

Gas chromatography-olfactometry (GC/O)

Ten grams of grated cheese (or 16 pieces of 2.5 cm^2 wrapper) were placed into a 40 mL amber screw cap vial (Supelco) to equilibrate for 30 min at 40 °C. The fiber was manually inserted into the sample vial at 2 cm depth for 30 min, followed immediately by desorption in the GC/O injector for 5 min at 250 °C. Prior to extraction, the fiber was conditioned in a GC injector for 5 min at 250 °C to prevent contamination.

Samples were analyzed on a HP5890 series II gas chromatograph (Hewlett-Packard Co., Palo Alto, Calif., U.S.A.) fitted with a flame ionization detector (FID), splitless injector, and a sniff port. The GC was equipped with either a polar capillary column (BD-WAX, 30 m length × 0.25 mm i.d. × 0.25 μm film thickness d_f; J&W Scientific), or a nonpolar column (DB-5 ms, 30 m length × 0.25 μm film thickness d_f; J&W Scientific). The oven temperature was programmed to increase from 40 to 200 °C at a rate of 10 °C/min with an initial hold of 3 min and a final hold of 20 min. Column effluent was split 1:1 between the FID and sniffing port using deactivated fused silica capillaries (1 m length × 0.25 mm i.d.). The FID and sniffing port were kept at 250 °C. Humidified air was supplied at the end of sniffing port at a rate of 30 mL/min to prevent dehydration of the nasal membranes of the panelists. Two experienced panelists (each with > 100 h experience with GC-O of dairy products) sniffed each sample in duplicate on the 2 different columns. The panelists described the odors and scored aroma intensities using a 5-point numerical intensity scale.

Identification and quantification of alkylmethoxypyrazines

Positive identification of 2-sec-butyl-3-methoxypyrazine and 2-isopropyl-3-methoxypyrazine in cheese was performed by comparison of volatile compounds mass spectra, retention index, and aroma quality with those of standard compounds analyzed under the same conditions. For the calculation of retention indices (RI), an n-alkane series was used (Van den Dool and Kratz 1963).

Standard curves were generated using cheese model systems, as described by Whetstine and others (2005). Mild Cheddar cheese served as the matrix because it neither contained EBP flavor nor the alkylmethoxypyrazines of interest, as determined by preliminary sensory and GC/MS analysis. Mild Cheddar cheese was shredded and portioned into several 25 g samples. Alkylmethoxypyrazines were first dissolved in methanol as stock solutions and then added to individual cheese shred aliquots to achieve concentration ranges between 10 and 150 ppb. In preliminary studies, these levels provided similar peak areas for 2-sec-butyl-3-methoxypyrazine and 2-isopropyl-3-methoxypyrazine in Farmstead Cheddar cheese containing EBP flavor. Cheese samples were kneaded by hand for 5 min, shaped into spheres and equilibrated for 24 h at 4 °C. A 6-point internal standard curve was constructed for each alkylmethoxypyrazine by plotting the peak area ratio of the analyte to 2-ethylpyrazine (internal standard) compared with the concentration ratio of analyte to 2-ethylpyrazine. Resultant peak area was fitted using linear regressions, with the concentrations averaged across 3 replications. GC/MS sensitivity was defined as the minimum concentration of an analyte that allowed accurate and reproducible determination of the peak height or area. The limit of detection for each alkylmethoxypyrazine was determined in a model system as the concentration that produced a "peak height-to-baseline noise" of at least 3 (signal-to-noise ratio $\geq$ 3).

Threshold testing

Orthonasal detection thresholds (that is, the lowest concentration at which an odor can be detected) were determined for 2-sec-butyl-3-methoxypyrazine and 2-isopropyl-3-methoxypyrazine using a 7-series forced choice ascending concentration method of limits (ASTM 1992). Threshold values were determined in both deodorized water and whole milk. Deodorized water was obtained by boiling 4 liters of distilled water to one-third of its volume. Whole milk was purchased from a local grocery store (Food Lion; Raleigh, N.C., U.S.A). From one row to the next, the stimulus concentration was increased by a factor of 3. Alkylmethoxypyrazine compounds were diluted in methanol and appropriate amounts of the stock solutions were added to samples. For each row, blank samples were adjusted with the same concentration of methanol to eliminate the effect of solvent. Samples were equilibrated at room temperature for 1 h before being presented for sensory evaluation in 58 mL lidded plastic soufflé cups labeled with a 3-digit code. Sample cups within each row were presented in random order.

Participants ($n = 40$) were instructed to sniff samples from left to right and choose the odd sample among the three. The participants were also asked to indicate judgment certainty (sure/not sure) within each row. The individual best estimate threshold (BET) was taken as the geometric mean of the last concentration with an incorrect response and the 1st concentration with a correct response with no further incorrect responses. If the participant indicated "not sure" for the correct choice, that concentration was adjusted by a factor of 1.41 to account for guessing (Lawless and others 2000). The group threshold was calculated as the geometric mean of the individual best estimate thresholds.

Sensory evaluation of cheese models

Sensory analysis of model systems was conducted to confirm that 2-sec-butyl-3-methoxypyrazine and 2-isopropyl-3-methoxypyrazine contributed to EBP flavor found in Farmstead Cheddar cheeses. Samples were evaluated using the same protocol applied for descriptive sensory analysis of cheeses.

Cheese model samples were prepared using a modified procedure described by Whetstine and others (2005). Alkylmethoxypyrazine compounds were first dissolved in ethanol as stock solutions. Mild Cheddar cheese was shredded and portioned into 5 samples of 250 g. Stock solutions were added to four of these samples to attain 2 and 20 ppb for 2-sec-butyl-3-methoxypyrazine and 0.4 and 4 ppb for 2-isopropyl-3-methoxypyrazine. These concentrations were all above the orthonasal thresholds determined in water and whole milk. The control sample contained ethanol in place of standard solutions. Each of the 5 samples was kneaded by hand for 5 min and shaped into a sphere. Samples were equilibrated for 24 h at 4 °C, and re-shaped into small spheres (8 g) prior to sensory evaluation. The samples were equilibrated for 1 h at room temperature prior to sensory analysis.

Statistical analysis

Data from descriptive sensory and instrumental analyses were evaluated by analysis of variance (ANOVA) using the general linear models procedure of SAS (version 9.1; Cary, N.C., U.S.A.). Means separation was performed using the Fisher's least significant difference (LSD) test.

Results and Discussion

Descriptive sensory analysis

The presence of EBP flavor was confirmed by trained panelists in 4 out of 8 Farmstead Cheddar cheeses (cheeses nr 1, 2, 7, and 8) (data not shown). Intensities of EBP flavor in the inner and outer sections ranged from approximately 1 to 3.5 on a 15-point scale. Previous studies have demonstrated that most cheese flavors fall between 0 and 6 on this scale (Drake and others 2005, 2008). The remaining 4 Farmstead Cheddar cheeses did not exhibit EBP flavor in any of the sections.

Characteristic descriptive profiles of inner and outer sections of Farmstead Cheddar cheese nr 1, purchased on 2 different occasions approximately 1 y apart were similar (Table 2). Cheeses were characterized by typical aged/developed flavors such as sulfur, brothy, and nutty. This is in agreement with the flavor profiles previously reported by Drake and others (2001) and Suriyaphan and others (2001) for British Farmstead Cheddar cheeses. EBP flavor was detected in both of these cheeses, with trends toward higher intensities for the outer sections as compared to the inner sections. Cheeses purchased and tested after 1 y showed larger differences in EBP flavor across sections, with sensory scores being 0.8 and 3.4 for the inner and outer sections, respectively (Table 2).

Identification and quantification of alkylmethoxypyrazines

Volatile compounds responsible for EBP flavor in Farmstead Cheddar cheeses were identified by SPME GC/MS and GC/O as 2-isopropyl-3-methoxypyrazine and 2-sec-butyl-3-methoxypyrazine (Table 3). Positive identification was achieved by comparison of volatile compound mass spectra, retention index, and aroma quality with those of standard compounds analyzed under the same conditions. Among 20 aroma active-compounds that were consistently identified in the cheeses by SPME GC/O, those alkylmethoxypyrazines were the only 2 compounds described as EBP at the sniffing port (data not shown). These results suggested that 2-isopropyl-3-methoxypyrazine and 2-sec-butyl-3-methoxypyrazine were likely the major compounds contributing to the EBP aroma found in Farmstead Cheddar cheeses.

Suriyaphan and others (2001) identified 2-isopropyl-3-methoxypyrazine and 2-isobutyl-3-methoxypyrazine as the sources of EBP flavor in a single British Farmstead Cheddar cheese. In the present study, the former compound was also identified as one of the contributors to EBP flavor. However, 2-sec-butyl-3-methoxypyrazine, rather than 2-isobutyl-3-methoxypyrazine, was identified as the 2nd source of EBP flavor in Farmstead Cheddar cheeses. In addition to similar chemical structures, 2-sec-butyl-3-methoxypyrazine and 2-isobutyl-3-methoxypyrazine share 2 out of the 3 largest ion peaks in their mass spectra ($m/z = 124$ and 151). These 2 compounds also have similar aroma profiles and retention indices (Table 3). Therefore, they could be misidentified as one another if sufficient precautions were not taken. In the present study, 2-sec-butyl-3-methoxypyrazine was positively identified in place of 2-isobutyl-3-methoxypyrazine by extracting the largest ion peak ($m/z = 138$) and comparing the mass spectra of eluting compounds to that of a 2-sec-butyl-3-methoxypyrazine standard. The procedure was repeated for 2-isobutyl-3-methoxypyrazine ($m/z = 124$); however, none of the eluting compounds had a similar mass spectrum as the respective standard. The more sensitive mass selective detector used in this study allowed a more accurate distinction between these 2 alkylmethoxypyrazines compared to the earlier study conducted by Suriyaphan and others (2001).

Quantification of 2-sec-butyl-3-methoxypyrazine and 2-isopropyl-3-methoxypyrazine in the inner, outer, rind, and wrapper sections was performed using internal standard curves generated with model systems. Linear correlation coefficients (R^2) for calibration curves were 0.99 and 0.97 for 2-sec-butyl-3-methoxypyrazine and 2-isopropyl-3-methoxypyrazine, respectively. The instrumental limit of detection was determined as 1 ppb for both alkylmethoxypyrazine compounds, with signal-to-noise ratios > 3. Quantification of these aroma components was achieved by solid-phase microextraction (SPME) and gas chromatography/mass spectrometry (GC/MS). Previous studies have shown that SPME is a powerful technique for extraction of 2-alklyl-3-methoxypyrazines in several complex matrices such as wine, fresh chili, and bell pepper (Hartmann and others 2002; Sala and others 2002; Mazida and others 2005).

Table 2—Flavor profiles of inner and outer sections of Farmstead Cheddar cheese nr 1 purchased on 2 different occasions approximately 1 y apart.

	Jan 2007[a]		Jan 2008[a]	
	Inner	Outer	Inner	Outer
Earthy/bell pepper	1.3a	1.7b	0.8a	3.4b
Cooked/milky	2.5a	3.0b	2.2a	2.2a
Milk fat	2.8a	3.3a	3.0a	2.5b
Sulfur	2.0a	2.0a	2.0a	2.5b
Brothy	3.4a	3.0b	3.0a	2.8b
Nutty	1.3a	1.5b	2.5a	1.3b
Sweet	2.3a	2.0a	2.5a	2.5a
Salty	3.5a	4.0b	3.5a	3.6a
Bitter	1.5a	1.0b	1.3a	1.0a
Sour	3.3a	3.3a	3.0a	3.0a
Phenolic	1.8a	1.5a	2.3a	1.0b
Umami	3.0a	3.3a	2.0a	2.0a
Prickle	—	—	1.8a	1.0a

[a]For each sample (Jan 2007 and Jan 2008), means in a row followed by different letters are different ($P < 0.05$).
[b]ND = not detected.

Both 2-sec-butyl-3-methoxypyrazine and 2-isopropyl-3-methoxypyrazine were prevalent towards the exterior sections of Farmstead Cheddar cheese nr 1. The concentrations of these compounds in the rind and wrapper sections were reproducible across 3 different samples of this cheese purchased and tested in a 1-y interval (Table 4). In general, concentrations of 2-sec-butyl-3-methoxypyrazine (150 to 444 ppb) were higher than 2-isopropyl-3-methoxypyrazine (39 to 115 ppb) in the wrapper ($P < 0.05$). The opposite trend was observed for the rind sections, where concentrations of 2-isopropyl-3-methoxypyrazine (557 to 730 ppb) were higher than those of 2-sec-butyl-3-methoxypyrazine (106 to 187 ppb) ($P < 0.05$). These alkylmethoxypyrazines were not detected by GC/MS in the inner and outer sections of any of the 3 samples of Farmstead Cheddar cheese nr 1.

Alkylmethoxypyrazines of interest were also present in the 2 samples of Farmstead Cheddar cheese nr 2 purchased and evaluated within a 1-y interval (0.5-kg cheese truckle and 2-kg cheese wedge from a 28-kg drum) (Table 5). Similar to results for cheese nr 1, 2-sec-butyl-3-methoxypyrazine was prevalent in the wrapper (162 ppb), and 2-isopropyl-3-methoxypyrazine was mostly found in the rind section (155 ppb) of the small truckle. Small concentrations of those alkylmethoxypyrazines were found in the inner and outer sections (Table 5). Compared to the small truckle, the cheese wedge, obtained from a large drum, had significantly higher concentrations of 2-sec-butyl-3-methoxypyrazine and 2-isopropyl-3-methoxypyrazine in the rind section (303 and 1200 ppb, respectively) ($P < 0.05$). Those alkylmethoxypyrazines were not detected in the inner and outer sections of this cheese (Table 5). Deviation of the distribution of alkylmethoxypyrazines across sections of the small truckle and cheese wedge (obtained from a large drum) may be explained by differences in their total size. During ripening, alkylmethoxypyrazines produced on the surface of the cheeses tend to reach the center of the small truckle before the center of the cheese wedge from the large drum because they have to travel a shorter distance. This may explain why those alkylmethoxypyrazines were detected in the interior sections of the small truckle but not in the cheese wedge.

Detectable levels of 2-isopropyl-3-methoxypyrazine were found in the rind section of the remaining Farmstead Cheddar cheeses as follows: cheese nr 7 wedge (32 ± 29 ppb), cheese nr 8 truckle (111 ± 120 ppb), and cheese nr 8 wedge (70 ± 28 ppb). 2-isopropyl-3-methoxypyrazine was not found in inner and outer sections, and 2-sec-butyl-3-methoxypyrazine was not detected in any section of these cheeses. Instrumental analysis of the remaining Farmstead Cheddar cheeses (cheeses nr 3, 4, 5, and 6) did not detect 2-sec-butyl-3-methoxypyrazine and 2-isopropyl-3-methoxypyrazine in any section of the cheeses. This finding was consistent with descriptive sensory analysis results that these cheeses did not exhibit EBP flavor.

In an earlier study (Suriyaphan and others 2001), the concentration of 2-isopropyl-3-methoxypyrazine was reported to be higher in the outer sections than at the center of a British Farmstead Cheddar cheese, which is in agreement with our results. The distribution pattern of pyrazine compounds in these cheeses suggests that the compounds are formed on or near the surface of the cheese and migrate toward the center during ripening. In the present study, Farmstead Cheddar cheeses had typical visible mold growth on the rind section, which suggests that EBP flavor related alkylmethoxypyrazines might be formed as microbial metabolites from mold naturally occurring on the cheese exterior. Several studies have shown that alkylpyrazines can be formed via the primary and secondary metabolism of microorganisms (Cheng and Reineccius 1991; Beck and others 2003). For example, 2-isopropyl-3-methoxypyrazine has been identified as a metabolite of *Pseudomonas perolans* and *Pseudomonas taetrolens* (Morgan 1976; McIver and Reineccius 1986; Gallois and others 1988; Cheng and Reineccius 1991). Beck and others (2003) identified several alkylmethoxypyrazines, including 2-isopropyl-3-methoxypyrazine and 2-secbuty-3-methoxypyrazine, as metabolites of *Paenibacillus polymyxa*. Brie and Camembert are cheeses that are surface-ripened by *Pencillium* sp., and their characteristic musty-earthy flavor has been linked to 2-isopropyl-3-methoxypyrazine produced by these fungi (Kosikowski 1982; Karahadian and others 1985b).

Threshold testing

Threshold values in water and whole milk were 1 ± 0.8 ppt and 16 ± 1 ppt for 2-sec-butyl-3-methoxypyrazine, and 0.4 ± 0.6 and 2.3 ± 1.1 ppt for 2-isopropyl-3-methoxypyrazine, respectively. Lower threshold values obtained for 2-isopropyl-3-methoxypyrazine in both matrices suggest that this compound is a more potent odorant than 2-sec-butyl-3-methoxypyrazine. Previous studies have reported threshold values for 2-isopropyl-3-methoxypyrazine in water ranging from 0.2 ppt (Young and others 1996) to 1.0 ppt (Murray and others 1970). A threshold concentration of 1 ppt in water has been reported for 2-sec-butyl-3-methoxypyrazine (Murray and others 1970; Fors 1988). Recently, Pickering and others (2007) reported that the ortho- and retronasal thresholds for 2-isopropyl-3-methoxypyrazine in 3 wine styles ranged from 0.32 to 2.29 ppt. Wagner and others (1999) determined the odor threshold for 83 alkylpyrazines in air and classified 2-alkyl-3-methoxypyrazines as a special class of odorants due to their relative low threshold values. They suggested that odor perception was a function of both steric and electrostatic interactions with receptors. Threshold concentrations for 2-isopropyl-3-methoxypyrazine and 2-sec-butyl-3-methoxypyrazine in air were 2 and 3 ppt, respectively. Many factors contribute to differences in the threshold values published in the literature. Those include testing method, delivery systems, number of

Table 3—Published and experimental values of retention indices (RI) and odor characteristics for selected alkylmethoxypyrazines.

	RI				Odor character	
	Experimental[a]		Literature[b]			
	DB-5MS	C20M	DB-5MS	C20M	Experimental[c]	Literature[b]
2-isopropyl-3-methoxypyrazine	1180	1410	1097	1427	Earthy, bell pepper, potato	Pea, earth
2-sec-butyl-3-methoxypyrazine	1094	1567	1176	1500	Potato, earthy, green	Carrot, earth
2-isobutyl-3-methoxypyrazine	ND[d]	ND	1186	1510	ND	Earth, spice, green pepper

[a]Retention indices (RI) were calculated from GC-O analysis.
[b]Available at http://www.flavornet.org.
[c]Odor character was determined by GC/O analysis.
[d]ND = not detected.

Table 4—Concentrations of alkylmethoxypyrazines in Farmstead Cheddar cheese nr 1 purchased on 3 different occasions within 1 y.

		Concentration (ppb)[a]			
		Inner	Outer	Rind	Wrapper
2-sec-butyl-3-methoxypyrazine	Jan 2007	ND[c]	ND	108 ± 44a[b]	444 ± 208b
	July 2007	ND	ND	106 ± 98a	150 ± 73a
	Jan 2008	ND	ND	187 ± 52	NA[d]
2-isopropyl-3-methoxypyrazine	Jan 2007	ND	ND	705 ± 118a	39 ± 5b
	July 2007	ND	ND	730 ± 345a	115 ± 110b
	Jan 2008	ND	ND	557 ± 116	NA

[a] Means for Jan 2007 samples were obtained from 4 cheese wedges (7 kg). Means for July 2007 and Jan 2008 samples were obtained from 1 cheese wedge (2 kg).
[b]Means followed by different letters are significantly different across cheese sections.
[c]ND = not detected.
[d]NA = wrapper not available at purchase.

Table 5—Concentrations of alkylmethoxypyrazines in Farmstead Cheddar cheese nr 2 purchased on 2 different occasions approximately 1 y apart.

		Concentration (ppb)[a]			
		Inner	Outer	Rind	Wrapper
2-sec-butyl-3-methoxypyrazine	Jan 2007	16 ± 15a[b]	8 ± 11a	16 ± 15a	162 ± 64b
	Jan 2008	ND[c]	ND	303 ± 93	NA[d]
2-isopropyl-3-methoxypyrazine	Jan 2007	5 ± 4a	29 ± 12a	155 ± 72b	33 ± 14a
	Jan 2008	ND	ND	1200 ± 480	NA

[a]Means for Jan 2007 samples were obtained from small truckle (0.5 kg). Means for Jan 2008 samples were obtained from a cheese wedge (2 kg).
[b]Means followed by different letters are significantly different across cheese sections.
[c]ND = not detected.
[d]NA = wrapper not available at purchase.

panelists, dilution steps, odorant type, and solvent used (Meilgaard and others 1999; Tsukatani and others 2003). Although most of the previously reported threshold values were not obtained using the ASTM method, they fell in the same range as experimental threshold values found in our study.

The exceptionally low threshold values of these alkylmethoxypyrazines indicate that they might contribute to flavor of Farmstead Cheddar cheeses despite the low concentration found in some of these cheeses. For example, alkylmethoxypyrazines were not detected toward the interior regions of Farmstead Cheddar cheese nr 1 by instrumental analysis. The limit of detection of the GC/MS instrument was determined as 1 ppb for both compounds, which is approximately 1000 times higher than detection thresholds in water and whole milk. Although detection thresholds of alkylmethoxypyrazines in cheese are expected to be higher than those determined in water and whole milk due to increased interactions between odorants and the more complex cheese matrix, descriptive sensory analysis showed that the inner and outer sections of Farmstead Cheddar cheese nr 1 exhibited EBP flavor (Table 2). This result indicates that the concentration of alkylmethoxypyrazines in cheese was above their detection thresholds but below instrumental limit of detection, which suggests that human perception of these aroma compounds was more sensitive than instrumental analysis.

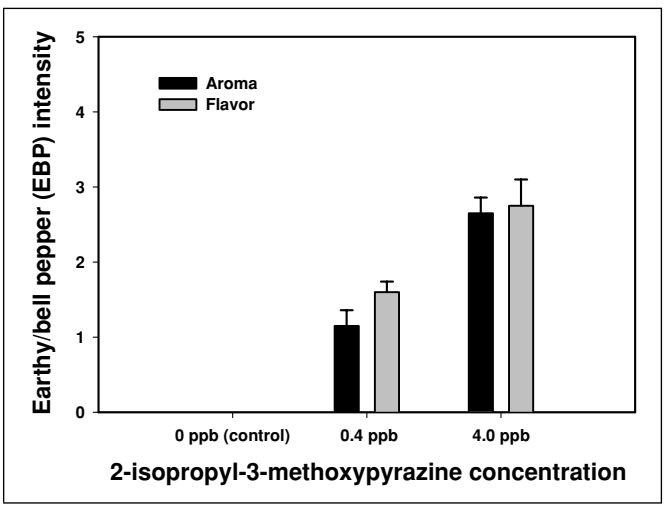

Figure 2—Impact of the addition of 2-isopropyl-3-methoxypyrazine on flavor and aroma of model cheeses. Error bars represent the standard deviation of panel means from duplicate analyses.

Model systems

The odor characteristics of 2-sec-butyl-3-methoxypyrazine and 2-isopropyl-3-methoxypyrazine were described as earthy/bell pepper/potato as they eluted from the GC/O column (Table 3). However, the individual aroma of a given compound may not reflect the actual role in flavor due to matrix interactions (Drake and Civille 2003; Singh and others 2003). Thus, assessing the effect of volatile components in a matrix similar to the food that is being studied is important. The contribution of 2-sec-butyl 3-methoxypyrazine and 2-isopropyl-3-methoxypyrazine to EBP flavor of cheeses was confirmed by sensory evaluation of model systems consisting of a mild domestic Cheddar cheese spiked with the pyrazine compounds. The addition of each pyrazine compound resulted in EBP flavor in cheese model systems (Figure 2 and 3). The panelists agreed that the EBP flavor resulting from addition of pyrazine compounds to cheeses was similar to that naturally found in Farmstead Cheddar cheeses. They also found no difference in the quality of EBP flavor resulting from addition of either pyrazine compound. However, 2-sec-butyl-3-methoxypyrazine required a concentration 5-fold higher than 2-isopropyl-3-methoxypyrazine to impart a similar sensory response, which was consistent with our threshold results that the latter is a more potent odorant.

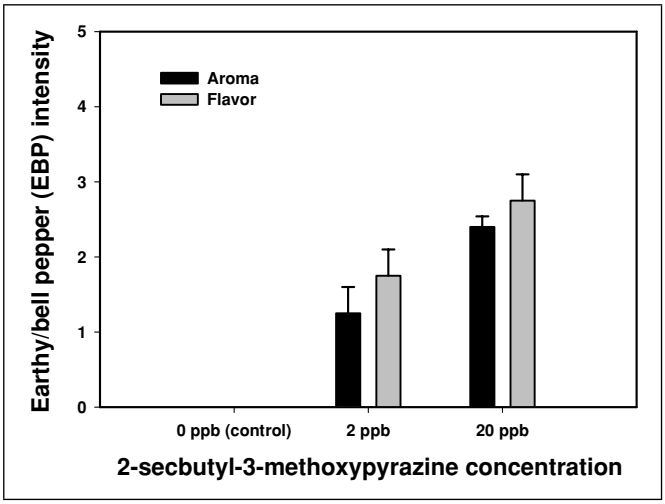

Figure 3—Impact of the addition of 2-sec-butyl-3-methoxypyrazine on flavor and aroma of model cheeses. Error bars represent the standard deviation of panel means from duplicate analyses.

Conclusions

This study revealed the importance of 2-sec-butyl-3-methoxypyrazine and 2-isopropyl-3-methoxypyrazine as the sources of EBP flavor in bandage wrapped Farmstead Cheddar cheeses. In general, alkylmethoxypyrazines were most prevalent in the cloth wrapper and the rind section of the cheeses. The 2 alkylmethoxypyrazines were either not detected in inner and outer sections of the cheeses or they were present at low concentrations. Despite the low concentrations of 2-sec-butyl-3-methoxypyrazine and 2-isopropyl-3-methoxypyrazine present in these sections, their contribution to flavor was significant because of their extremely low threshold values. The results of this study suggest that alkylmethoxypyrazines causing EBP flavor are possibly formed as metabolites of microorganisms present on or near the surface of the cheese and that the compounds migrate into the cheese during ripening. Formation of EBP flavor in Cheddar cheese seems to require a natural rind exposed to the air during ripening/aging rather than vacuum sealing in polyethylene bags as is done for most commercial Cheddar cheeses. This may explain why EBP flavor is only found in certain Farmstead Cheddar cheeses with natural air-exposed rinds.

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